ACTION AND TRANSMISSION SPECTRA OF PHYCOMYCES[1,2]

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In this paper measurements are presented of action spectra for the photoresponses of sporangiophores of Phycomyces and of the transmission spectra of the growing zone of single sporangiophores in stage IV b (7) and of the cell wall. Compared to other organisms, Phycomyces offers several definite advantages for such measurements. There are two distinct photoresponses, the growth response and the tropic response, and there are many indications, particularly the action spectra presented here, that these two responses are mediated by the same pigment. The growth response occurs under conditions of symmetric irradiation when the intensity varies with time, and the tropic response occurs with an irradiation program which may be constant in time, but asymmetric with respect to azimuth angle, i.e., the angle around the axis of the sporangiophore. Crudely speaking, the growth response measures the azimuthal summation of photoeffects, while the tropic response measures the azimuthal asymmetry. The combination of these two measurements permits an estimate of the influence of screening pigments lying *between* the receptors proximal to the incident light and those distal to it. Similarly, the measurement of transmission spectra of the intact sporangiophore and of the cell wall permits some conclusions with respect to the absorbing materials present in various locations.

Another advantage of Phycomyces is that the optics of the system are relatively simple. The sensitive region closely approximates a cylindrical lens with uniform refractive index. The importance of the dioptric properties of this lens for the tropic effect was clearly demonstrated 40 years ago by Buder (2), who showed that immersion of the specimens in a medium with refractive index higher than that of the cell contents reversed the sign of the tropic response. Thus, the dioptric properties, both refractive and reflective, and not scattering or absorption have the decisive influence in producing the azimuthal asymmetry in the sporangiophore.

Two years ago, Curry and Gruen (10) discovered that the tropic sensitivity of the sporangiophores extends deep into the ultraviolet region and that the tropic response reverses sign around 300 mμ. The reversal has a simple explanation. There are screening substances in the growing zone which, for wavelengths shorter than 300 mμ, effectively shield the distal side of the sporangiophore from the incident light. For these short wavelengths, then, the sporangiophore can be stimulated truly unilaterally, in contrast to the visible region, where both sides are stimulated, the distal more than the proximal, when the sporangiophore is irradiated unilaterally.

METHODS

CONCEPT OF "VISUAL" PIGMENT Any response of an organism to light may be expected to involve at least one substance which undergoes a *photochemical reaction* which ultimately, through a more or less complicated chain of events, leads to the observable response. This substance will be referred to here as the "visual" pigment. The general purpose of making measurements of action spectra is to obtain information regarding the absorption spectrum of this visual pigment. However, the action spectrum *of the effect* may differ from the action spectrum *of the pigment* because of the intervention of screening pigments or of self screening. These difficulties will be discussed after presenting the data. The action spectrum of the pigment is usually equated with its absorption spectrum. This equation is not necessarily valid, since the quantum yield may vary with wavelength.

EQUAL RESPONSE VERSUS EQUAL FLUX ACTION SPECTRA Visual pigment absorbs different wavelengths with different absorption coefficients. This variability may be exhibited either as a difference in the *response* at various wavelengths under conditions of equal energy or quantum flux, or as a difference in *flux* needed to produce equal responses. Both methods have been used by various authors and both are likely to disclose at least the gross features of the absorption spectrum. The equal response method, however, seems preferable for quantitative work because it does not involve the uncertainty of translating differences in response into differences in amount of the primary photochemical product. The measurements presented here are based entirely upon the principle of equal response.

NULL VERSUS FINITE RESPONSE The next question concerns the choice of size of the "equal response". If there were no perturbations from side effects, such as screening, any size response should give the same action spectrum. The perturbations show up in a different dependence of the response on the flux at different wavelengths. A clear example of this may be found in the work of Shropshire and Withrow (16)

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on the action spectrum of phototropic tip-curvature of Avena. Such perturbations can be interpreted properly only if a great deal of information is available besides the action spectrum. Quite aside from these perturbations, the question of the amount of response which should be chosen to obtain the most accurate measurements still remains. One condition is that the response should vary as strongly as possible with the flux. Another condition, well known in physical measurements, is that the response should vary symmetrically with the flux. This condition is best realized if the response is a null response, varying symmetrically between negative and positive values for small deviations from the null. Such a method eliminates perturbations arising from variability in sensitivity between specimens.

GROWTH RESPONSE NULL DETERMINATIONS For growth response the condition of null response was realized by irradiating the specimen during alternate equal intervals of 5 minutes from a standard light source and a monochromatic source. If these two sources appear equally bright to the specimen, there will be no growth response. This is the null condition. If the standard light appears brighter than the test light, there will be a periodic growth response whose maximum occurs about 7.5 minutes after the standard light is turned on; i.e., in the middle of the test light period. If the test light appears brighter, there also will be a periodic growth response, but with a phase shift of 180°.

The symmetry of the irradiation was assured by rotating the specimen continuously at 2 rpm. The arrangement for doing this while maintaining accurate positioning has been described previously (9). The speed of 2 rpm is sufficiently fast to eliminate tropic effects and sufficiently slow to avoid mechanical problems. Figure 1 illustrates the irradiation program and the actual responses from minute to minute under various conditions of relative size of the test and standard stimulus. The method by which the flux producing the null response was interpolated from a series of such measurements is illustrated in figure 2 and explained in the figure legend.

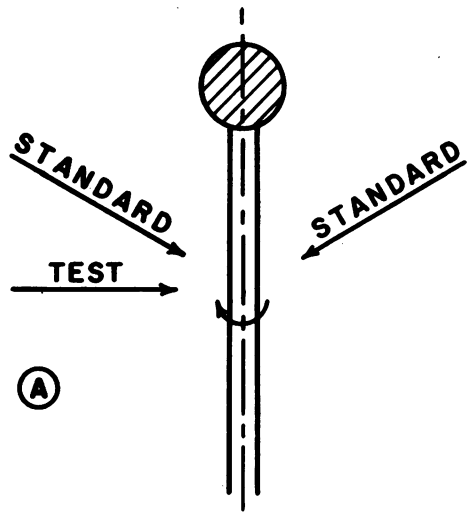
TROPIC NULL DETERMINATIONS For the tropic response the null method is more obvious and has been used by previous authors[4] (5). The standard light impinges from one side and is matched against the monochromatic test light. The monochromatic light impinges from the other side, if test and standard produce tropic effects of the same sign, and from the same side, if they produce tropic effects of opposite sign. It is important that the two beams of light do not come in at an angle of 180° from each other, but at a lesser angle. If the angle is 180° and the sources are of equal effectiveness, the specimens are indifferent phototropically; i.e., they are at equilibrium in whichever direction they grow (11). Another condition, only imperfectly realized in these experiments, is that the irradiation ought to be symmetric with respect to the vertical in order to eliminate perturbations from geotropism (12). Arrangement of the light sources is shown diagrammatically in figure 3. The equilibrium intensity of the test beam was determined by changing the intensity until the equilibrium position was obtained and maintained for 3 hours under continuous irradiation. Variations in the direction of growth of a single specimen during constant irradiation and variations between specimens amount to $\pm$ 10°. This variation sets a limit to the accuracy in a single determination of the matching quantum flux of $\pm$ 10 %. The matching quantum flux was determined for one to three sporangiophores at each wavelength. The sensitive zone of each sporangiophore was continuously centered to within $\pm$ 2 mm in all directions from the center of the field to eliminate the effect of small intensity gradients in the field.

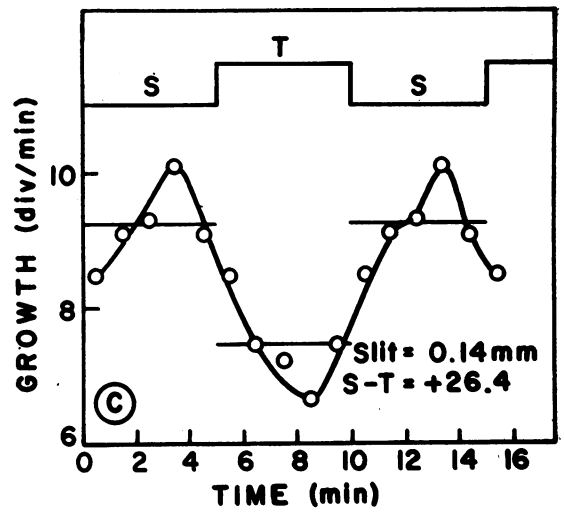
EQUIPMENT FOR STIMULATION The standard stimulus was provided by a tungsten lamp with a Corning heat filter [1-69] and a Corning blue filter [5-61] arranged in tandem. Arrangement of the light source, stage, microscope, etc., as well as the intensity scale, have been described in detail previously (11). For most of the measurements the intensity of this irradiation was I = −8. Unilateral I = −8 corresponds in effectiveness to a monochromatic flux at 440 mμ of 0.022 μw/cm².

The test stimulus was provided by a uniform field (1.5 × 2.0 cm) of monochromatic flux obtained 30 cm from the exit slit of a Beckman DU spectrophotometer. Only the light sources and the dispersing elements of this instrument were used. The tungsten lamp was used for wavelengths longer than 330 mμ and the hydrogen lamp for shorter wavelengths. The intensity of the monochromatic flux is proportional to the square of the slit width (since entrance and exit slit vary in parallel) and this intensity control, after verifying its validity, was used in preference to neutral filters. The *maximum* half-intensity bandwidth, as calculated from Beckman instrument data, was 5 mμ. In most cases it was considerably smaller. The wavelength calibration was checked by isolating the bright lines from a medium pressure mercury arc source. The test light impinged horizontally from one side only, while the standard light impinged 60° from the vertical, from one or both sides.

CALIBRATION OF ENERGY FLUX The plate current, suitably amplified, of a calibrated RCA 935 phototube was used to measure the monochromatic flux. The sensitivity of the phototube was determined from 240 to 405 mμ by the Experimental Tube Division of RCA, using the strong mercury lines. For longer wavelengths the tube was calibrated by comparison with a Kipp thermopile and Leeds and Northrup galvanometer system, which in turn had been calibrated against a secondary irradiation standard. From the sensitivity data the quantum fluxes for a 0.10 mm slit were tabulated at 5 mμ intervals.

[4] See addendum


STANDARD
STANDARD
TEST
A


S
T
S
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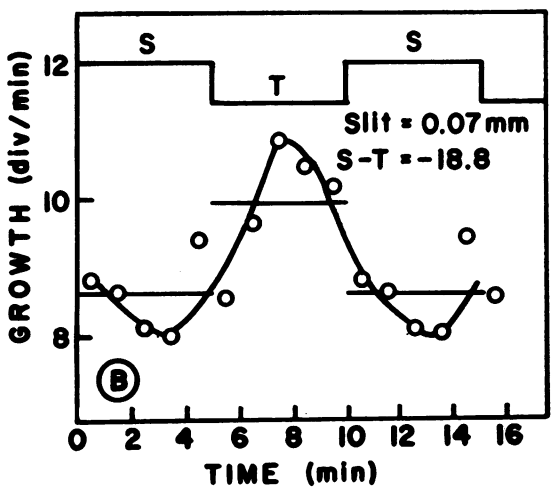
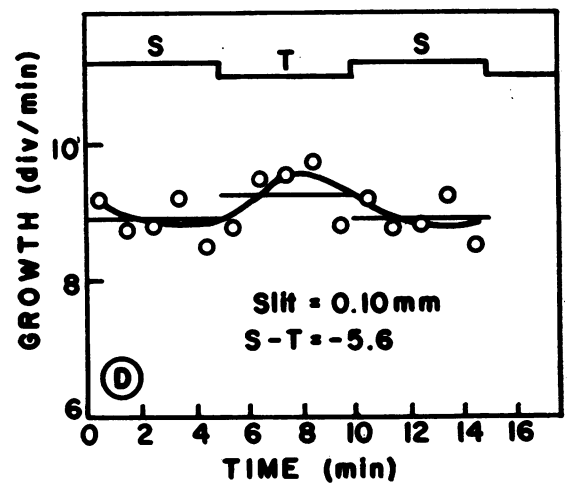
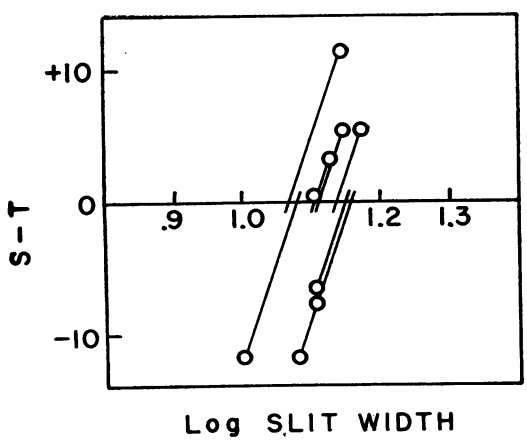
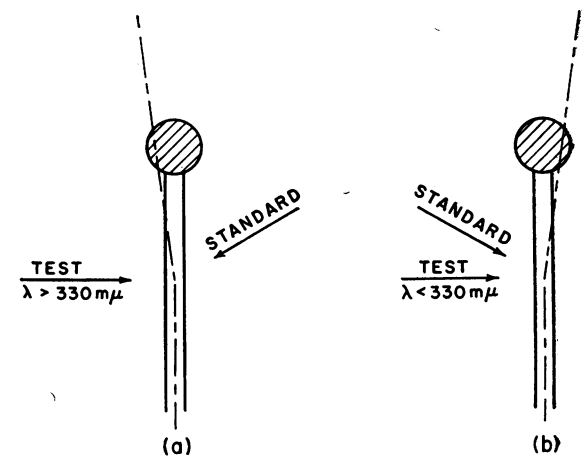
8
6
Slit = 0.14mm
S-T = +26.4
C
0 2 4 6 8 10 12 14 16
TIME (min)


S
T
S
GROWTH (div/min)
12
10
8
Slit = 0.07mm
S-T = -18.8
B
0 2 4 6 8 10 12 14 16
TIME (min)


S
T
S
GROWTH (div/min)
10
8
6
Slit = 0.10mm
S-T = -5.6
D
0 2 4 6 8 10 12 14 16
TIME (min)


+10
0
-10
S-T
.9
1.0
1.2
1.3
Log SLIT WIDTH


TEST
λ > 330mμ
STANDARD
(a)
STANDARD
TEST
λ < 330mμ
(b)

TRANSMISSION SPECTRA *Mounting specimens* After determining the action spectra, which showed pronounced peaks, an attempt was made to detect the visual pigment directly by the absorption of light during its passage across the sporangiophore. The dioptric properties of the specimens in air make it difficult to arrange a suitable geometry of the light paths in the optical system. These difficulties are much reduced by immersing the specimens in a medium of matching refractive index which is also transparent and non-toxic for the specimen. Pure fluorochemical FC-43, perfluorotributylamine, (Minnesota Mining and Manufacturing Co.) closely approximates these conditions. Specimens completely submerged in it grow at a normal rate for many hours and give normal growth responses. Its refractive index is 1.29 which is close to that of the cell contents (1.34 from our measurements).

It is convenient to work with sporangiophores which have been severed from their mycelium in order to mount them on a photographic plate or a microscope stage. Such specimens retain their turgor for many hours but do not grow when mounted in air or FC-43. Gruen (15) discovered that isolated sporangiophores grow and respond normally for many hours when their base is immersed in water. We have found that when the growing zone is in contact with water, growth ceases quickly and turgor is lost. Thus, for the present purposes, it was desirable to have specimens with their feet in water and their tops in FC-43. Microchambers (figure 6a) were constructed with two partitions, separated by silicone grease. An isolated sporangiophore is placed with the top half in one partition, where it is immersed in FC-43, and the lower half in the other partition, where it is immersed in water. Such specimens grow at a normal rate of 3 mm/hr with little or no lag due to shock of immersion, give normal growth responses, and can be observed continuously under a high power microscope. This arrangement permits a check whether or not the transmission of non-growing isolated specimens differs from that of growing specimens. No differences were found. Thus, the non-growing specimens give a valid comparison picture to the action spectra, which naturally require growing specimens.

Numerous methods for the transmission measurements were tried and the two relatively most successful ones are presented. For both methods the specimens were immersed in FC-43.

SHADOWGRAPH METHOD The specimen is placed directly on the emulsion of a photographic plate and irradiated from above with nearly parallel monochromatic light. The shadowgraph thus produced is evaluated densitometrically. Since the specimens are only 0.1 mm in diameter and the plates have to be scanned across the specimen, this method requires fine grain emulsion and high resolution densitometry. The method is laborious in requiring a new specimen for each wavelength and densitometric calibrations for each wavelength setting. It is particularly useful in the ultraviolet since no optics are involved in taking the shadow graphs; the method is informative with regard to the amount of light scattered by the cell contents under various angles.

Turgid, isolated sporangiophores were placed on Kodak medium lantern slide plates, imbedded in FC-43, covered with quartz coverslips supported by 0.5 mm spacers, and exposed to monochromatic flux. FC-43 can be left in contact with a photographic emulsion for long periods without affecting it. Exposures were such that the half-intensity bandwidths were 5 mμ or less and the background blackening was at the top of the linear range for a plot of optical den-

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FIG. 1 (*upper 2 rows*). Growth response, null method. A. Directions of irradiation. The specimen is rotated at 2 rpm to make the irradiation azimuthally symmetric. B, C, and D. Growth speeds during successive 1 min intervals. Above each response curve the irradiation program is indicated. The points in the response curves represent averages over three successive cycles. Average values during the first half of the cycle are repeated to facilitate the drawing of a smooth curve through the points. In B, with the slit of the monochromator at 0.07 mm, the test light is weaker than the standard light. The response curve has a minimum near the middle of the standard irradiation. In C, with the slit of the monochromator at 0.14 mm, the test light is stronger than the standard light. The maximum of the response occurs near the middle of the standard irradiation. In D, with a slit of 0.10 mm, the two irradiations are nearly matched. To evaluate the response, the total elongations during each of three standard (S) and three test (T) periods were determined. The difference between these two quantities, S-T, is negative in B, positive in C, and ideally zero at the growth response null. This condition is approximated in D. The horizontal lines are the average growth speeds during standard and test periods.

FIG. 2 (*lower left*). Interpolation method for determining the growth response null. The quantity S-T, obtained as explained in figure 1, is plotted versus the logarithm of the slit width. From the total of all measurements at all wavelengths it was found that S-T near the null varies linearly with the logarithm of the slit width, with a slope 3. Assuming this relation, every experimental point in this graph may be used to estimate a slit width giving null response. These estimates were averaged for five to ten sporangiophores at each wavelength to estimate the matching quantum flux.

FIG. 3 (*lower right*). Arrangement of the light sources for determining the tropic null. The dotted line indicates the direction of equilibrium. Due to geotropism, this direction is slightly closer to the vertical than the bisector of the two directions of growth produced by each of the sources separately.

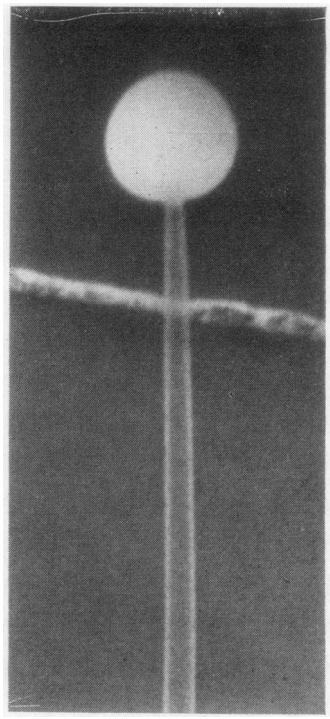


FIG. 4. Shadowgraph method for determining transmission spectra. The specimen, immersed in FC-43, was placed directly on the emulsion of the photographic plate and irradiated with a parallel beam of monochromatic light. The shadowgraph was obtained at 380 mμ and is enlarged 45 x. Light bands at the margins of the sporangiophore are due to the residual difference between the refractive indices of the medium (1.29) and the cell contents (1.34). The residual lens effect produces an increased intensity in the center of the shadowgraph calculated to be 14 %.

sity (O. D.) versus the logarithm of exposure. Exposures were varied by changing exposure times at constant intensity. The gamma (γ) of the lantern slides and suitable exposure times were determined for the range from 240 to 540 mμ, in 10 mμ steps over most of the range and 5 mμ steps near the maxima, minima, and inflection points of the action spectra. The plates were scanned 1 to 2 mm below the sporangium with a microdensitometer capable of measuring fields 1.2 × 25μ (figs 4 and 5). The same instrument was used to determine the γ of the plates.

Below 300 mμ so little light is transmitted (due to absorption) that background and specimen cannot be compared in a single photograph. For these points longer exposures were made until the transmitted light gave appreciable blackening and the O.D. was estimated by comparing the exposure times used with those needed to produce similar background blackening. At very high O.D.'s this procedure is subject to errors from scattering, so that only lower limits of the O.D. can be estimated.

DIRECT METHOD In the second method the total transmitted light is measured *directly*. A 2 mm section of the exit slit of the monochromator is imaged with a 5 in focal length lens on the growing specimen and is carefully aligned parallel to the specimen axis. A slit width is chosen such that the image of the slit is narrow compared to the sporangiophore diameter. The light paths are therefore collimated to nearly the same degree as in the shadowgraph method. The total transmitted light (the undeviated and a large proportion of the scattered light) is collected by a microscope objective and measured by a photometer whose sensitive element intercepts the image of the observed section of the specimen. This method is convenient since the entire wavelength range can be scanned within a few minutes. It is also more accurate than the shadowgraph method since it does not require elaborate calibrations at each wavelength and avoids interspecimen variability. This gain is offset, however, by extreme requirements in alignment, focusing, positioning and by chromatic aberrations in the optics. These factors introduce errors which seem to be particularly bad in the near-ultraviolet. The method could not be used at all below 400 mμ.

The specimen, contained in a microchamber on the mechanical stage of a Reichert microscope, was aligned using the regular microscope optics (fig 6b). The eyepiece and top part of the tube were then removed and the image produced by a 45x objective projected via a totally reflecting prism onto the sensitive surface of a 1P21 photomultiplier tube of an Eldorado photometer. The specimen was moved in and out of the beam by fine motions of the stage controlled by reproducible front and back stops. At each wavelength setting the background was adjusted to zero by varying the fine sensitivity control of the photometer and the O.D. of the specimen in the growing zone alone or below the growing zone was determined directly by moving it into the beam. Under these conditions any light transmitted directly or scattered within the specimen into a forward angle less than about 45° was intercepted by the objective lens (N.A. = 0.65) and projected onto the sensitive surface.

RESULTS

GROWTH RESPONSES AND ADAPTATION AT DIFFERENT WAVELENGTHS The question of whether or not the specimens exhibited any qualitative differences in their responses to different wavelengths, aside from the reversal of phototropism, was examined first. Figure 7 shows that the growth responses elicited by stimuli of different wavelengths are exactly comparable, even deep in the ultraviolet where the stimulating light penetrates only a short distance into the specimen. Furthermore the ultraviolet is comparable to the visible in its adapting effects. As is well known (11), growth responses depend upon the ratio of the stimulus size to the level of adaptation, and the latter, at equilibrium, is proportional to the intensity of the adapting light. Light of any wavelength

was found to be interchangeable for either stimulus or adaptation, providing the intensity was adjusted by the calibration factor corresponding to the growth response action spectrum.

TROPIC RESPONSES AT DIFFERENT WAVELENGTHS In contrast to the growth response there are gross qualitative changes in the tropic response at different wavelengths. The most obvious of these is the reversal of the sign of the tropic response below 300 mμ.

In the visible a short unilateral stimulus of *moderate* size produces a tropic response for which the time of onset is very nearly the same as that of the growth response (3). A *strong* unilateral stimulus produces at best a feeble and somewhat delayed tropic response. This phenomenon was discovered by Castle (4) and named *phototropic indifference*. Castle interpreted it by assuming that a strong stimulus gives a saturated growth response on both the proximal and the distal side and, therefore, no differential between the two sides.

Below 300 mμ, the distal side is shielded. Under these conditions *any* stimulus capable of producing a growth response should also give a tropic response. This is indeed the case. Below 300 mμ a strong, short unilateral stimulus gives a strong, transient, negative tropic response. The response begins around 4 minutes after the stimulus and results in a maximum tilt of 70° or more. The maximum *rate of tilting* is about 8°/min and occurs at 5 to 7 minutes after the stimulus. The *maximum tilt* occurs at 11 to 15 minutes and the tilt is back to zero at about 30 minutes. These are precisely the characteristics expected for a strictly unilateral growth response.

Similarly, continued unilateral stimulation at 280 mμ gives a continued negative tropic response, with a maximum rate of tilting greater than 20°/min.

For specimens immersed in FC-43 the match of refractive indices is so close that no phototropic response occurs even after prolonged unilateral irradiation with blue light. Irradiation with wavelengths shorter than 300 mμ, however, produces a strong negative tropic reaction. This is to be expected, since the specimen is stimulated exclusively on the side proximal to the impinging light.

GROWTH RESPONSE AND TROPIC RESPONSE ACTION SPECTRA Figure 8 shows the results of these measurements, obtained by the null method described above. The matching intensity was the same (or a small multiple) in all cases, $I = -8$; i.e., close to the lower end of the "normal range" (11). For each wavelength measured, the ordinate gives the reciprocal quantum flux which matches the standard light. For wavelengths below 300 mμ the matching reciprocal quantum fluxes for the tropic responses are entered as negative values to indicate that the test light impinged from the same side as the standard light.

TRANSMISSION SPECTROSCOPY OF GROWING ZONE As pointed out above, the shadowgraph method was the only one available below 400 mμ. This method was also used for longer wavelengths but was less precise than the direct method. The precision for the shadowgraph method was ± 0.1 O.D. unit, while for the direct method the values were reproducible for a given specimen to within 0.01 O.D. unit. In figure 9 the measurements in the ultraviolet are given both for intact sporangiophores and for cell walls from which the contents had been squeezed out.

The O.D. rises sharply slightly above 300 mμ (13). Dennison has identified the principal substance in the sap which absorbs in the ultraviolet as gallic acid. Since the onset of absorption of this substance agrees with our O.D. findings, the absorption curve of gallic acid, adjusted to equal O.D. at 290 mμ is given in figure 9. The match between the two curves is remarkably good above 250 mμ. It is poor below 250 mμ, where the expected O.D. of gallic acid decreases while the observed O.D. continues to remain high. Presumably, other substances contribute to the O.D. in this region.

The concentration of gallic acid needed to produce the indicated O.D. in a pathlength equal to the diameter of the sporangiophore (100 μ) is about 5 mg/ml. This is close to the value estimated by Dennison for the concentration of gallic acid in the extracted *sap* of *whole* sporangiophores (13). These facts strongly suggest that the internal screen, at least near the neutral point, is gallic acid.

For later discussion of the inversion of phototropism the O.D. contributed by gallic acid in the critical region between 300 and 330 mμ is given in table I.

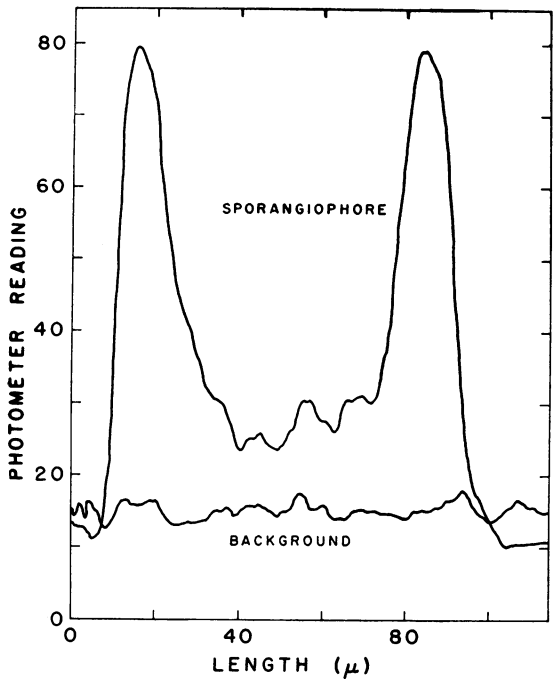
The absorption of the cell wall (fig 9) shows the absorption peak reduced by a factor of about ten. This is believed to be due to residual contents not eliminated during the squeezing process.

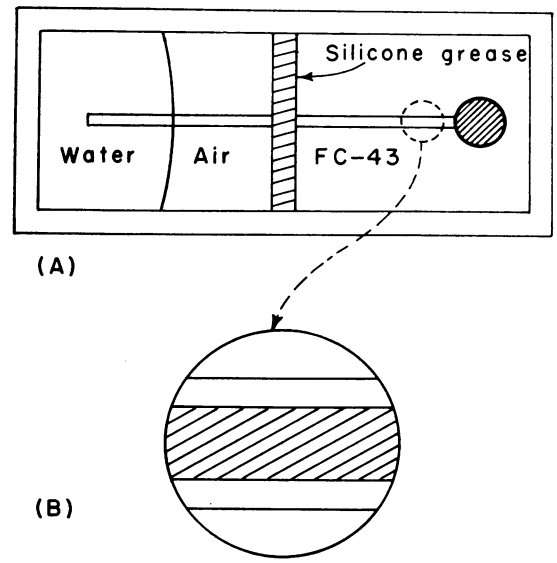
In the visible the results were quite variable, and are therefore not plotted. A few fairly regular features could be discerned, however. Generally, a background O.D. of about 0.1 was found. This is presumably due in large part to nonspecific absorption and in small part to reflection losses and residual scattering not collected by the microscope objective. Superimposed on this background is a slight absorption, amounting to a maximum O.D. of about 0.03, beginning at about 530 mμ, and giving maxima around 500, 460 and 430 mμ. In sporangiophores grown on a lactate medium, this characteristic absorption is much stronger, contributing in some cases O.D.'s between 0.1 and 0.2. It is likely that the substance con-

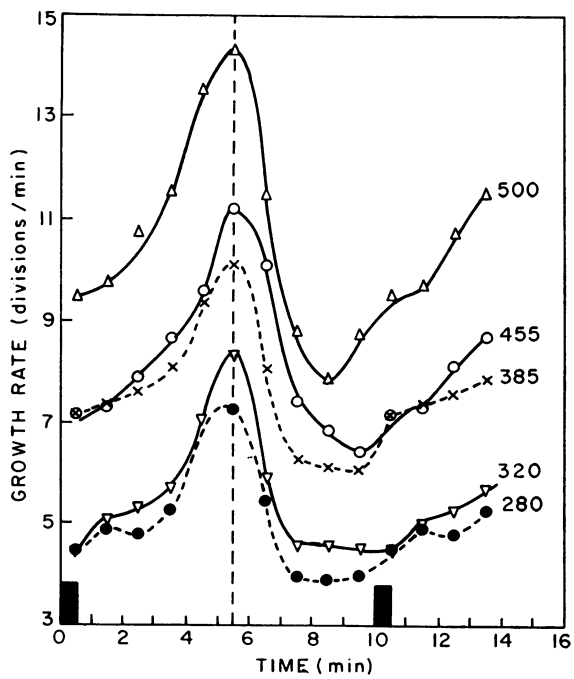
TABLE I

OPTICAL DENSITY OF GALLIC ACID (5MG/ML FOR 100 μ PATHLENGTH)

λ	O.D.
300	0.70
310	0.24
320	0.05
330	0.006







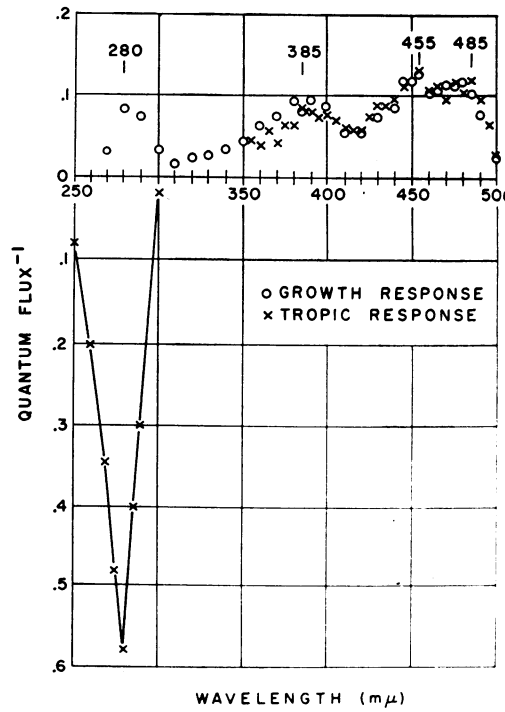


FIG. 5 (*upper left*). Microdensitometer tracing (1 mm below the base of the sporangium) across the sporangiophore of the original shadowgraph of figure 4. Irregularities in the tracing are due to grain in the emulsion. The principal point exhibited is the sharpness of the edge of the sporangiophore, indicating that scattering does not affect the optics of the sporangiophore appreciably.

FIG. 6 (*upper right*). Microchamber for direct transmission measurements. A. Top view of chamber. B. The field imaged by the microscope objective onto the sensitive element of the photometer, showing the image of the slit of the monochromator (hatched area) in relation to the specimen.

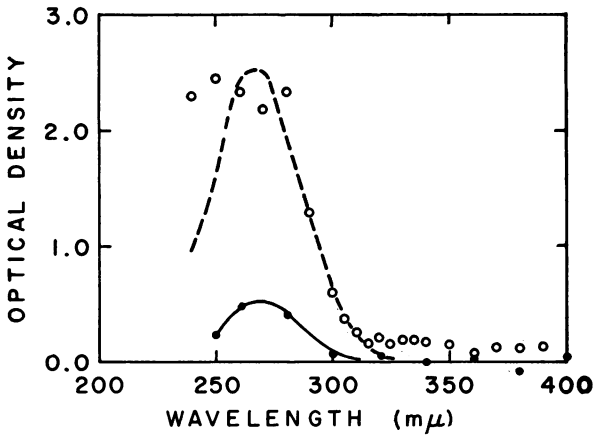


FIG. 9. Transmission spectra of the growing zone of the sporangiophore and of the primary cell wall in this region. The dotted line is the optical density of gallic acid (5 mg/ml 100μ pathlength). Open circles denote the O.D. of intact growing zone and solid circles the transmission curve of the cell wall with imperfectly extruded cell contents. The data are corrected for the lens effect mentioned in the legend to figure 4.

tributing these three peaks is β-carotene, since β-carotene can be isolated from the extracts and has similar absorption peaks, except for a slight wavelength shift.

The cell wall had no characteristic absorption (less than 1 %) in the visible and near-ultraviolet, even when the specimens had been carefully dark adapted before extruding the cell contents.

No bleaching by blue light could be observed, even when the specimen was growing and responding normally and had been thoroughly dark adapted.

DISCUSSION

The principal aim of this discussion is to determine what can and cannot be concluded with respect to the action spectrum of the visual pigment. The following are the main points in our data:

I. The growth and tropic action spectra are identical from the upper limit at 500 mμ to 360 mμ, and show peaks at 385, 455, and 485 mμ.

II. Below 300 mμ the tropic action spectrum is a negative image of the growth action spectrum, enlarged at the peak (280 mμ) by a factor 6.5.

III. The cell wall does not show a characteristic absorption.

IV. Beyond 400 mμ the O.D. of the growing zone of a sporangiophore, whether non-growing, or growing and reactive, whether dark adapted or not, shows no obvious peaks which could be correlated with those of the action spectra.

V. The O.D. begins to rise sharply near the inversion point and reaches a maximum (about 2.5) at the 280 mμ peak.

These findings are discussed in terms of the following postulates which are somewhat weaker than those originally proposed by Blaauw (1):

Growth responses are transient and occur as a result of *changes* in the intensity of irradiation. A positive intensity transient (a step-up or a pulse-up) produces a positive growth response. Any phototropic effect in Phycomyces is conditioned by a *bilateral asymmetry* of light energy *absorbed.* (A puzzling feature of the tropic response to continuous unilateral irradiation is that it is not transient, like the growth response to a step-up in the intensity, but persists beyond the time at which the growth response transients disappear. This has been discussed in detail by Cohen and Delbrück (8)).

For evaluating the effectiveness of light absorbed we have to take into account its precise azimuthal distribution (6). Thus, for unilateral irradiation in air where the total light absorbed on the proximal side probably slightly exceeds the amount absorbed on the distal side, the distal absorption is nevertheless more effective due to the advantage conferred upon the distal side by the focusing effect. We have no a priori measure of the magnitude of this advantage, but will attempt to evaluate it from our data. Let us say that the focusing effect in air gives the distal side the stimulus 1+a when the proximal side receives unit

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FIG. 7 (*lower left*). Growth responses at different wavelengths. The specimens were rotated at 2 rpm and continuously adapted by irradiation with standard light. They were stimulated every 10 min for 30 sec with monochromatic light of suitable intensity. The points are averages for three cycles. The growth responses obtained at different wavelengths are similar. Maximum response occurs at 5.5 min after the stimulus. The response time is independent of the average growth rate and of the size of the response. The correlation between wavelength and average growth rate of the specimen is accidental.

FIG. 8 (*lower right*). Action spectra for growth response and tropic response. For the tropic response data, the matching quantum fluxes were determined with a precision of ± 10 %. The precision of the growth response data was somewhat better. The maxima at 280 and 385 mμ and a broad region of high sensitivity between 440 and 490 mμ are unambiguous. The dip between the two maxima at 445 and 485 mμ could be due to a combination of experimental errors and screening effects, as explained in the text. Otherwise the action spectra, where they coincide, are believed to represent faithfully the action spectrum of the visual pigment. The distortions below 300 mμ are due to internal screening by gallic acid. The growth response peak at 280 mμ is low by *at least* a factor two, as compared to the peak in the action spectrum of the visual pigment (see text).

stimulation. Superimposed on this advantage is a factor, 1−b, accounting for any attenuation of the light in its passage across that part of the sporangiophore between the proximal and the distal receptors. Such an attenuation could be due to the visual pigment itself and to other substances. The focusing advantage a is presumably independent of wavelength (neglecting dispersion), while the attenuation b depends strongly on the wavelength. In the visible the attenuation is small compared to unity, according to point 4 above. Since the reversal occurs between 320 and 310 mμ, where the total O.D. is still small compared to unity and the absorption due to gallic acid contributes an O.D. between 0.05 and 0.24 (see table I), we conclude that the focusing advantage is also small compared to unity. In the visible, then, both a and b are small and we can neglect squared terms. Thus, the ratio of the effects on the two sides is

$$\text{distal : proximal} = (1+a-b) : 1.$$

The effects of various types of screening on the two action spectra may now be considered systematically.

I. Internal Screen, Attenuation Slight, $b<<1$ The growth action spectrum will not be affected, since it compares the sum of the effects on proximal and distal sides, standard versus test. Screening and focusing contribute only second order correction terms.

In contrast, the tropic action spectrum may be affected, since the null condition requires that the differentials are matched. Let the quantum flux of the standard light and also the absorption coefficient of the visual pigment for this light be unity. Let the quantum flux of the test light at equilibrium be x and the absorption coefficient of the visual pigment for this light be β. Let b_s and b_λ be the attenuations of standard and test light. Then the effects on the two sides will be:

standard side	$1 + \beta\, x(1+a-b_\lambda)$
test side	$\beta\, x + 1+a-b_s$

For tropic equilibrium, these two expressions must be equal. This gives for the action spectrum 1/x the expression

$$1/x = \beta\,(a-b_\lambda)/(a-b_s).$$

This equation says that the action spectrum and the absorption spectrum of the screening pigment will be in an inverse relation to each other. The action spectrum (1/x) will have a dip where the screening pigment (b_λ) has a maximum. The reason for this is obvious: the screen increases the *intensity* differential where its spectrum has a maximum; it thereby *reduces* the advantage conferred upon the distal side by the focusing effect.

This effect is appreciable only where b_λ is comparable to a. In our data the effect is obvious as the inversion point is approached. As the O.D. of the screen rises and becomes comparable to the focusing advantage the tropic action spectrum drops off to zero.

Equally significant is the fact that we find no such screening effect in the visible as evidenced by the strict parallelism of the two action spectra (point 1). This indicates that there is no internal screening affecting appreciably the advantage due to focusing.

Self-screening by the visual pigment, if it is slight, has qualitatively similar effects. It, too, will not affect the growth action spectrum, but will affect the tropic action spectrum inversely, when it is comparable to the focusing advantage. Again we can conclude from the parallelism of the two action spectra that self-screening in the visible is negligible compared to the focusing advantage (less than 10 % of the latter). In sum, we conclude that the internal attenuation across the sporangiophore in the visible, both due to self- and foreign-screening, is negligible.

II. Internal Screen, Attenuation Strong, $1\text{-}b<<1$ The growth action spectrum will be affected by a factor two, since the standard light acts on both sides while the test light reaches the proximal side only. To match the standard light, therefore, the test light has to provide a quantum flux twice higher than corresponds to the absorption coefficient of the visual pigment, and the action spectrum is low by a factor two compared to the visual pigment absorption spectrum.

For the tropic action spectrum the situation is quite different. Consider a tropic equilibrium when visible light impinges from the same side as light of, say, 280 mμ. The visible light acts at the rate one on the proximal side and 1+a on the distal side. The ultraviolet acts on the proximal side only. To balance the asymmetry in the stimulation produced by the standard light it must act at the rate a. Thus, in such a combination, the ultraviolet does not actually match the standard light in its direct effect on the visual pigment, but only compensates the differential of this effect across the sporangiophore. Therefore, the quantum flux needed for tropic balance is low by the factor a, and the action spectrum high by the factor 1/a.

Combining these two corrections we are now in a position to estimate a numerical value for the focusing advantage a. To do this we must raise the growth action value by the factor two and compare it then to the tropic action value, which is 1/a times larger. This gives for a the value 0.3.

III. External Screens Any screening substance located entirely external to the photoreceptors will affect the growth and the tropic action spectrum equally and increase the quantum fluxes needed to match the test stimulus. Thus the action spectra should show dips where the screens show peaks. Unfortunately, we do not know where the visual pigment is located and, therefore, also do not know which regions are to be considered external. The cell wall is certainly external and it does not show any characteristic absorption (point 3). However, this does not suffice as a justification for ignoring the possible

effects of external screens. It may well be that β-carotene and gallic acid lie, at least in part, external to the visual pigment. The distortions from this source could be appreciable. In the ultraviolet, if only 10 % of the gallic acid were located external to the photoreceptor it would cut off half the flux at 280 mμ and make both action spectra low by a factor two. In the blue, if all the blue absorbing pigments as measured by the total O.D. were entirely external to the photoreceptor, half on each side, the action spectra would be depressed by an O.D. of 0.015. This depression would affect the action spectra by 3 to 4 % at its absorption peaks and might be responsible for some of the dip in the action spectra separating the peaks at 455 and 485 mμ.

The principal conclusions regarding the action spectrum of the visual pigment of Phycomyces are the following:

In the visible and near-ultraviolet both action spectra give a rather faithful image of the visual pigment action spectrum, not distorted by internal, external, or self-screening. Distortions from internal screens are entirely negligible. External ones might give distortions of a few percent; hardly noticeable within the accuracy of our measurements. The only significant region that might be affected by this correction is the dip separating the maxima at 455 and 485 mμ.

The total absorption of light in its passage between the photoreceptors on the two sides of the sporangiophore must be less than 10 % of the focusing advantage. Since this is less than the directly measured attenuation across the whole sporangiophore and since the cell wall has no measurable O.D., we must infer that the measured attenuation is due to pigments located peripherally but inside the cell wall. This makes it seem likely that the photoreceptors are not located at the cell wall but some distance inside. Perhaps they lie at the interface between protoplasm and vacuole or at some interface inside the protoplasm.

Regarding the chemical nature of the visual pigment we can rule out β-carotene with some confidence, because of the peaks at 280 and 385 mμ in the action spectra. Various flavin compounds and other substances mentioned in the literature (14) whose absorption spectra show greater similarity with the action spectra are possible candidates. With the action spectrum given it would seem hopeful to isolate the visual pigment and identify it by chemical means. Our attempts in this direction have not yet been successful. The O.D. produced by visual pigment must be very small; it does not show up in the transmission measurements, and self-screening does not affect the differential across the sporangiophore responsible for the tropic reaction.

The discussion of the interrelationship between the action spectrum of the growth response and that of the tropic response illustrates how strongly both spectra, and particularly the tropic spectrum, can be modified by screening pigments. In Phycomyces there happens to be no appreciable distortion in the visible, though a very strong one below 350 mμ. In Avena the work of Shropshire and Withrow (16) has shown that the tropic action spectrum is strongly affected by screening pigments even in the visible. To correct for these screening effects, it would be necessary to have a clear notion of the cause and magnitude of the bilateral asymmetry in the absence of screening pigments, similar to the notion of the focusing advantage in the case of Phycomyces. We do not have such a theoretical basis for Avena. We suspect that, here, too, dioptric effects are involved, since Ziegler (17) has shown that the phototropism for Avena reverses sign when the specimens are immersed in paraffin oil. We have confirmed this effect and have found that the extreme tip of the coleoptile, where most of the sensitivity resides, is sufficiently transparent to permit dioptric effects to be operative. A detailed growth action spectrum would help clarify this question.

Summary

Detailed action spectra of the growth response and of the tropic response of the sporangiophores of Phycomyces in stage IVb are given for 10 mμ intervals from 250 to 300 mμ and for 5 mμ intervals from 300 to 500 mμ. Both spectra have been determined by null response methods. The two spectra are identical in the visible and near-ultraviolet and show maxima at 280, 385, 455, and 485 mμ. Below 300 mμ the tropic response reverses sign and shows a peak at 280 mμ. This peak is 6.5 times larger than a corresponding peak in the growth action spectrum.

Transmission spectroscopic data in the growing zone have been obtained for single sporangiophores. In the ultraviolet these were obtained from shadowgraphs and in the visible by direct photometric measurements. In the ultraviolet a strong absorption is found, beginning near the phototropic inversion point and paralleling the absorption of gallic acid, a substance which had previously been detected by Dennison in the vacuole. In the visible region, transmission, including most of the scattered light, is never more than about 75 %. Only a minute part of these transmission losses can be due to absorption by the visual pigment. Most of the losses must occur peripheral to the visual pigment, but not in the cell wall.

The growth responses are qualitatively identical throughout the spectrum. The tropic responses, besides reversing sign below 300 mμ, are also stronger in this region than in the visible. These facts, as well as the quantitative relationship between the two action spectra, can be explained satisfactorily as mediated by the screening effect of an internal screen which is principally due to gallic acid.

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Addendum

A paper by G. M. Curry and H. E. Gruen has just appeared (Proc. Natl. Acad. Sci. 45: 797. 1959) in which action spectrum data for the phototropic response of Phycomyces are presented. The gross features of the results of these authors are similar to ours. Differences in detail may be accounted for by the method used by Curry and Gruen, involving mass cultures and the determination of balance points between the standard and the test light source which are set up 2 meters apart. This method, which we had tested and rejected, involves two disadvantageous features apparently not appreciated by the authors:

I. Mass cultures lead to mutual shading, and this produces spurious balance points near the middle of any series of cultures set up together.

II. The intensity ratio received from the two sources varies with position as $(1+x)^2/(1-x)^2$, where x is the displacement from the center, taking the distance from the center to one of the sources as unit. This is a very rapid variation, approximately $1+4x$ for small displacements from the center. Thus, 2.5 cm displacement changes the ratio by 10 %. A glance at table I of the cited paper shows that balance points in repeat measurements differed in some cases by as much as eight times this amount.

In view of these criticisms, the contention of Curry and Gruen of a double peak in the blue, characteristic of the absorption of β-carotene, cannot be considered as substantiated by their measurements.

Literature Cited

1. Blaauw, A. H. 1909. Die Perzeption des Lichtes. Rec. trav. bot. neerl. 5: 209–372.
2. Buder, J. 1918. Die Inversion des Phototropismus bei Phycomyces. Ber. deut. bot. Ges. 36: 104–105.
3. Castle, E. S 1930. Phototropism and the light-sensitive system of Phycomyces. Jour. Gen. Physiol. 13: 421–435.
4. Castle, E. S. 1931. Phototropic "Indifference" and the light-sensitive system of Phycomyces. Bot. Gaz. 91: 206–212.
5. Castle, E. S. 1931. The phototropic sensitivity of Phycomyces as related to wave-length. Jour. Gen. Physiol. 14: 701–711.
6. Castle, E. S. 1933. The physical basis of the positive phototropism of Phycomyces. Jour. Gen. Physiol. 17: 49–62.
7. Castle, E. S. 1942. Spiral growth and the reversal of spiraling in Phycomyces and their bearing on primary wall structure. Amer. Jour. Bot. 29: 664–672.
8. Cohen, R. and M. Delbrück 1959. Photoreactions in Phycomyces: Growth and tropic responses to the stimulation of narrow test areas. Jour. Gen. Physiol. 42: 677–695.
9. Cohen, R. and M. Delbrück 1958. Distribution of stretch and twist along the growing zone of the sporangiophores of Phycomyces during normal growth and during periodic illumination. Jour. Cellular Comp. Physiol. 52: 361–388.
10. Curry, G. M. and H. E. Gruen 1957. Negative phototropism of Phycomyces in the ultra-violet. Nature 179: 1028–1029.
11. Delbrück, M. and W. Reichardt 1956. System analysis for the light growth reactions of Phycomyces. Cellular Mechanisms in Differentiation and Growth. Rudnick, Ed. Princeton University Press. Pp. 3–44.
12. Dennison, D. S. 1958. Studies on phototropic equilibrium and phototropic-geotropic equilibrium in Phycomyces. Thesis, California Institute of Technology.
13. Dennison, D. S. 1959. Gallic acid in Phycomyces sporangiophores. Nature 184: 2036.
14. Galston, A. 1959. Phototropism of stems, roots and coleoptiles. Handbuch der Pflanzenphysiologie 17(1): 492–529.
15. Gruen, H. 1959. Growth and development of isolated Phycomyces sporangiophores. Plant Physiol. 34: 158–168.
16. Shropshire, W., Jr. and R. B. Withrow 1958. Action spectrum of phototropic tip-curvature of Avena. Plant Physiol. 33: 360–365.
17. Ziegler, H. 1950. Inversion phototropischer Reaktionen. Planta 38: 474–498.